the gel with 10% phosphate-10% citrate (pH 7.6), while incubating at 30 to 35°C. The eluted liquid was dialyzed against an aqueous solution containing 0.1 M citrate. Then, the solution was loaded onto a column of DEAE-Sephadex and the toxin was eluted with phosphate buffer containing 0.1 to 0.2 M saline (hereafter abbreviated as PBS). Subsequently, the sample was loaded onto a column of Sephadex G-75 and the toxin was eluted with PBS. The resulting sample was subjected to gel electrophoresis. Only the presence of the cholera toxin as a single band was detected. The purity was about 95%. The yield was 250 mg/100L culture liquid.

Example 2 - Attenuation of cholera toxin:

The purified cholera toxin obtained in Example 1 was dissolved in 0.01 M PBS (pH 7-8). 0.05 M lysine was added to the solution. Then formalin was added dropwise to each aliquot of the solution at a final concentration of 0.1, 0.3, 0.5, 0.6, 0.8, or 1.0%. The resulting solutions were incubated at 30 to 40°C for 7 to 96 days. Samples were taken during the incubation. Immediately, the collected samples were dialyzed against 20 times as much volume of PBS to remove formalin. The dialyzed solution was then sterilized by filtration to give attenuated cholera toxin. The time course of the activity change was investigated by using part of the samples collected during the treatment. Figure 1 shows an example where the sample collected on the twelfth day of the formalin treatment was assayed by the method for the residual toxic activity, utilizing as an index the binding ability to ganglioside GM1, which is a receptor for cholera toxin B subunit (a subunit having the binding activity to target cells of toxin; A subunit is responsible for the toxic activity). The result shows that the binding ability of the attenuated toxin to ganglioside GM1 is reduced to about 1/15 and 1/100 of the natural one, when the toxin was treated with 0.3% and 0.5% formalin for 12 days, respectively.

The Y-1 cell morphologic transformation test was utilized to assay the residual toxic activity of a variety of samples that were subjected to attenuation treatment under various conditions. Part of the result is indicated in Table 2. The result shows that the residual activity is reduced by a factor of about 1/1780 to about 1/114000

when the toxin was treated with 0.3% and 0.5% formalin for 12 to 96 days. Acute toxicity test was conducted in mice by using the attenuated cholera toxin prepared above. The attenuated cholera toxin, indicated in Table 2, in which the residual toxic activity was 1/7000 of the original activity or lower, did not show toxicity when administered intraperitoneally at a dose of 30 mg/kg. Accordingly, the toxic activity can be reduced to one-two thousandth or lower (using acute toxicity in mouse as an index) by treating the toxin with 0.3% formalin at 35°C for 12 days or more.

Table 2

Residual toxic activity of formalin-treated cholera toxin (according to the result obtained by Y-1 cell morphologic transformation test)

Formalin Treatment		Residual Toxicity (ED50)		Attenuation Rate
Concentration (%)	Days	Dilution (4^n)	(4^n)	Approximate Value of Attenuation Magnification
0.3	12	2.0±0.6	6.7	10,800
	24	2.3±0.6	6.4	7,130
	48	2.7±0.6	6.0	4,100
	72	3.3±0.6	5.4	1,780
	96	3.3±0.6	5.4	1,780
0.5	12	0.3±0.6	8.4	114,000
	24	0.7±0.6	8.4	65,500
	48	1.3±0.6	7.4	28,500
	72	1.3±0.6	7.4	28,500
	96	0.7±0.6	8.0	65,500
No treatment control		8.7±0.6		1

Example 3 – Enhancing effect on the secondary production of antibody against influenza HA vaccine:

HA antigen prepared from influenza virus PR8 strain (A/Puerto Rico/8/34, H1N1 type) that had been habituated to mouse was used as an antigen for vaccination. Attenuated cholera toxin was used as the

adjuvant. The residual toxic activity of attenuated cholera toxin used was 1/1780 to 1/114000 that of the original activity (according to Y-1 cell morphologic transformation test). Five Balb/c mice (6-weeks-old, females) were used for each group in the test. The mice were anesthetized with sodium amobarbital, which was administered into the peritoneal cavity. 10 µl of PBS containing 1 µg vaccine antigen and 1 µg adjuvant was given dropwise to either nasal cavity of the mouse for intranasal immunization. Four weeks later, the secondary immunization was carried out in the same manner. Two weeks after the secondary immunization, sera and nasal washes were collected from the mice.

The titer of anti-influenza virus antibody in the serum was determined based on the HI antibody titer. After bloodletting, the nasal washes were collected from the mice by perfusing the right and left nasal cavities with 1 ml of PBS containing 0.1% bovine serum albumin (BSA). The quantities of anti-HA-IgA antibody in the nasal washes and anti-HA-IgG antibody in the sera were determined by enzyme immunoassay (ELISA). Prior to the assay for anti-HA-IgA, each well of EIA plate was treated with 50 µl of HA vaccine (5 µg/ml) suspended in a coating buffer. The plate was allowed to stand still for the coating at room temperature for 2 hours. The plate was then washed with PBS containing Tween-20 (hereinafter abbreviated as PBS-Tween). Subsequently, each well was coated with 100 µl of PBS containing 1% BSA and 0.1% NaN_3 to prevent unspecific reactions. The plate was allowed to stand still at 4°C overnight, and then washed with PBS-Tween. A 100-µl aliquot of adequately diluted nasal wash sample was added to each well. After several hours, the reaction solution was discarded and the well was washed with PBS-Tween. Subsequently, 100 µl of alkaline phosphatase-labeled goat anti-mouse IgA α chain-specific antibody (or alkaline phosphatase-labeled goat anti-mouse IgG antibody) diluted with PBS containing 1% BSA and 0.1% NaN_3 was dispensed into the respective wells. The plate was allowed to stand still at room temperature for an hour and then washed. Finally, p-nitrophenyl phosphate (1 mg/ml; Sigma Co.) dissolved in 10% diethanol amine buffer (pH 9.8) was added to each well for color development. The plate was allowed to stand still at room temperature for 20 to 30 minutes, and